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(71) Applicant: IAF BIOCHEM INTERNATIONAL INC. [CA/CA]; 2550 Daniel-Johnson Blvd., Suite 600, Laval, Quebec H7T 2L1 (CA).

(72) Inventor: LACROIX, Martial ; 9025 Richmond, Brossard, Quebec J4X 2R9 (CA).

(74) Agent: MORROW, Joy, D.; Smart & Biggar, 900-55 Metcalfe Street, P.O. Box 2999, Station D, Ottawa, Ontario K1P 5Y6 (CA).

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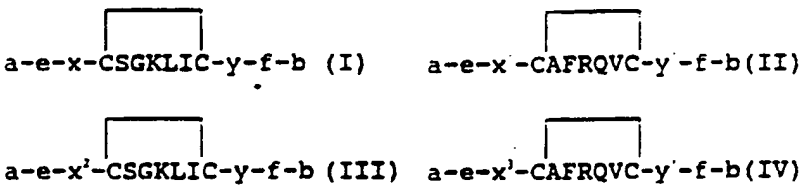
(54) Title: SYNTHETIC PEPTIDES AND MIXTURES THEREOF FOR DETECTING HIV ANTIBODIES



(57) Abstract

There is provided cyclic peptides of general formulae (I) and (III), wherein x represents an amino acid sequence from position 585 to 604 (gp41-HIV-1) such amino acid sequences being characterized by at least one of a lysine at position 586 or a lysine at both positions 585 and 586; x^2 represents an amino acid sequence from position 585 to 604 (gp41-HIV-1); y represents an amino acid sequence from position 612 to 629 (gp41-HIV-1); e and f represent one or more epitopes included in the amino acid sequence extending from 586 to 629 (gp41-HIV-1) or from 578 to 613 (gp36-HIV-2); and a and b represent the amino and carboxy terminals, respectively, as well as substituents which are effective to make the peptide more useful as an immunodiagnostic reagent. Peptides of formula III have one or both of e and f present. There is also provided peptides of general formulae (II) and (IV), wherein x^1 represents an amino acid sequence from position 577 to 596 (gp36-HIV-2) such amino acid sequences being characterized by at least one of a lysine at position 578 or a lysine at both positions 577 and 578; x^3 represents an amino acid sequence from postion 577 to 596 (gp36-HIV-2); y^1 represents an amino acid sequence from position 604 to 613 (gp36-HIV-2); e and f represents one or more epitopes included in the amino acid sequence from 578 to 613 (gp36-HIV-2) or 586 to 629 (gp41-HIV-1); and a and b are as defined above. Peptides of formula (IV) have one or both of e and f present. These cyclic peptides alone or in admixture with other peptides are particularly useful in detecting HIV-1 and HIV-2 antibodies in analytes and sera.

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SYNTHETIC PEPTIDES AND MIXTURES THEREOF FOR DETECTING HIV ANTIBODIES

This is a continuation-in-part of United States patent applications 07/148,821 filed on January 27, 1988, 07/185,518 filed April 22, 1988, and 07/281,205 filed December 8, 1988.

FIELD OF THE INVENTION

The present invention relates to novel cyclic peptides and combinations thereof alone and with linear and cyclic peptides for detecting HIV antibodies.

BACKGROUND OF THE INVENTION

Acquired Immune Deficiency Syndrome (AIDS), AIDS related complex (ARC) and pre-AIDS are thought to be caused by a retrovirus, the Human Immunodeficiency Virus (HIV). The first AIDS related virus, HIV-1 (also known as HTLV-III, LAV-1 and ARV) has been well characterized. Another pathogenic human retrovirus named HIV-2 (formerly LAV-2) has now been isolated from West African patients with AIDS. See, e.g., WO 87/04459. HIV-2 has recently been shown (Guyader et al. Nature 326 662-669, 1987) to share a number of conserved sequences with HIV-1 and the Simian Immunodeficiency Viruses (SIV).

Even though other numbering systems are used in the art, for ease of understanding and comparison we have adopted herein the amino acid numbering system of

Ratner et al., Nature, 313, 277-284, 1985 for the HIV-1 proteins and that of Guyader et al., Nature 326, 662-669 (1987) for the HIV-2 proteins. The amino acids in the peptides of this invention are designated by the single letter code as follows: ala=A, arg=R, asn=N, asp=D, cys=C, gln=Q, glu=E, gly=G, his=H, ile=I, leu=L, lys=K, met=M, phe=F, pro=P, ser=S, thr=T, trp=W, tyr=Y and val=V.

The initial immunodiagnostic tests for the detection of antibodies in the serum of patients infected with HIV-1 utilized the whole virus as antigen. Second generation tests made use of polypeptide sequences obtained by the recombinant DNA methodology. Cabradilla et al. Bio/Technology 4 128-133 (1985) and Chang et al. Bio/Technology 3, 905-909 (1985), for example, refer to bacterially synthesized viral protein fragments of 82 and 102 amino acid residues, respectively. EPA 202314 and 114243 refer to recombinant polypeptides spanning regions of gp41 and gp120 that are immunoreactive alone or in mixtures. Shoeman et al., Anal. Biochem. 161, 370-379 (1987) refers to several polypeptides from gp41 that are immunoreactive with antibodies present in sera from patients infected with HIV-1. None of the above assay procedures is, however, totally acceptable. Their lack of sensitivity is a critical shortcoming. It may permit blood containing virus to escape detection and thereby potentially result in the infection of blood product receivers and continued infectivity by undiagnosed AIDS carriers. Their lack of specificity (false positives) is also a problem -- healthy individuals are told they may have AIDS. Such false positive may be caused by impurities. They may also be caused by shared epitopes with viruses unrelated to AIDS present in th se antigen preparations. In this

r gard, Gallaher, Cell 50 327-328, 1987 has reported that a region of gp41 of HIV-1 shares a sequence of five adjacent amino acid residues with the respiratory syncytial virus and of four equally distributed amino acids of the measles virus F1 glycoprotein. Thus, even highly purified recombinant polypeptides containing this region, or any other common regions yet to be discovered, could potentially be responsible for false positives and the attendant unacceptable specificity. Finally, these prior art assays do not permit detection of very low levels of HIV antibodies. This disadvantages the assays in terms of their ability to detect AIDS infections at a very early stage, thereby delaying the start of treatment and permitting the possible spread of infection by blood samples and other body fluids before effective detection of AIDS infection.

In an attempt to solve these problems, diagnostic means and methods employing shorter HIV antigens are now being developed. Empirical methods to identify peptide sequences corresponding to unique and highly conserved epitopes of the HIV viruses are also now available. These methods are, for example, capable of assisting in the selection of short amino acid sequences which are more likely to be exposed on the surface of the native protein and thus useful as assay tools (for a review see Hopp and Woods, J. Immunol. Met. 88, 1-18, 1986). Although somewhat useful, these methods are no more than indicative. Nonetheless, they have been applied to identify epitopes present on the surface of viruses responsible for AIDS. For example, US Patent 4,629,783, International Patent Appl. PCT/US86/00831 and EPA 303224 refer to various synthetic peptides from the p18, p25, gp41 and gp120 proteins. These peptides are

advantag d by the relative ease and low r cost with which they can be prepared and more importantly because of the reduced risk of obtaining false positives with them due to impurities or the presence of shared epitopes with viral proteins not related to AIDS.

While these smaller peptides are advantaged in terms of specificity over the earlier recombinant polypeptide and whole virus approaches to the diagnosis of AIDS infections, they have been less than satisfactory in terms of overall sensitivity, perhaps because the synthesized epitope is not able to assume and maintain a conformation that is recognized by the AIDS antibodies. Although the number of serum samples tested in each of these cases is very limited, specificity (few if any false positives) was found to be very high (95%-100%) with the small synthetic peptides but the overall sensitivity varied between 80% and 100%. In fact, in the only example where 100% sensitivity was attained only ten samples were tested. For example, Smith et al., J. Clin. Microbiol. 25 1498-1504, 1987 refers to two overlapping peptides, E32 and E34, that are highly immunoreactive. No false positives, out of 240 seronegative specimens, were obtained but the peptides missed three seropositive samples out of 322 (sensitivity of 99.1%). Wang et al. (Proc. Natl. Acad. Sci 83, 6159-6163, 1986) refers to a series of overlapping peptides (including amino acid residues of Smith's E32 and E34 peptides) among which one 21-mer peptide showed 100% specificity and 98% sensitivity (out of 228 seropositive samples taken from patients with AIDS, 224 were found positive with this peptide). And United States patent application 120,027, filed November 13, 1987 refers to a short synthetic peptide spanning residues 606 to 620 (SGKLICTTAVPWNAS) of gp41 (HIV-1). This peptide is

said t be immunor active with antibodies of patients infected by the AIDS viruses. The specificity was also excellent (63/63) but 6 seropositive specimens out of 57 confirmed positive could not be detected (sensitivity of 89%).

Gnann et al. (J. Virol. 61, 2639-2641, 1987 and J. Infec. Dis 156, 261-267, 1987) also refer to a series of overlapping peptides from a suspected immunodominant region of gp41 (HIV-1). Gnann et al. concluded that cys-605 was essential for the immunoreactivity of that segment of the gp41-(HIV-1) protein. They reported that a peptide having the sequence SGKLIC (606-611) was not immunoreactive with any of the 22 HIV-1 positive sera tested, while the addition of the cysteine residue to the N-terminus restored some immunoreactivity, 21 of 44 sera reacted with the 7-mer peptide (48% sensitivity).

Gnann et al. (J. Virol) also speculated that the cysteine residues at positions 605 and 611 of gp41 (HIV-1) might play a role in the antigenic conformation of this region perhaps by the formation of a cyclic structure via disulfide bonding. However, Gnann et al. never demonstrated that they did have a synthetic peptide wherein the two cysteine groups were linked by disulfide bonds.

Although Gnann et. al refers to peptides which are useful in identifying HIV-1 antibodies, even its peptides lack 100% sensitivity. For example, Gnann et al. (J. Virol. 61, 2639-2641, (1987)) report that while their 600-611 amino acid sequence detected 22 out of 22 positive sera, they also reported that similar tests carried out at the Centers for Disease Control, Atlanta, Ga. with the same 12-amino acid sequence (600-611) missed 1 out of 79 positive sera. And Gnann et al. in J. Infect. Dis. 156, 261-267, 1987 report d

that the same 12-amino acid sequence was reactive with 131 out of 132 HIV-1 infected patients from the United States.

Gnann et al. Science 237, 1346-1349, 1987 reports a short linear synthetic peptide spanning residues 592 to 603 of gp42 (HIV-2) that contains two cysteines in a region homologous to the 605-611 region of gp41(HIV-1). This peptide reacted with 5 out of 5 sera taken from HIV-2 infected patients.

Other peptides containing amino acids 605-611 of gp41 of (HIV-1) are also referred to in the art. WO 86/06414 refers to peptide X(39), which is encoded by the region from about bp 7516 through bp 7593, and peptide XIII(79) which is encoded by the region extending from about bp 7543 through bp 7593, both containing the 7-amino acid sequence 605-611. These peptides are reported to be linear and no formation of cyclic structures is suggested. WO 87/06005 reports that a series of synthetic peptides encompassing the Cys(605)-Cys(611) residues of the HIV-1 envelope glycoprotein (gp41) undergo a series of spontaneous oxidative transformations upon solubilization in neutral or basic aqueous buffer. It speculates that as a result, the peptides when used in ELISAs are a random mixture of linear monomer, cyclic monomer, linear or cyclic dimers and linear polymers of various lengths. The application did not actualy demonstrate the presence of cyclic components and did not characterize the other various dimers and polymers possibly present. Moreover, it speculates that the polymer forms are the most important components for ELISA reactivity.

In addition to perhaps being complex mixtures of various oxidative forms of the peptide, the prior art peptides referred to above do not permit as early detection of AIDS infection as would be desirable. For

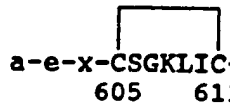
example, Gnann et al. (J. Virol) reports that when the HIV-1 positive sera are diluted by a factor exceeding 500, some of these diluted sera are found to be negative thus indicating a low sensitivity of the peptide for early HIV detection.

These problems have been addressed by employing peptides that have been chemically cyclized to form a disulfide bridge between the relevant cysteines. E.g., M. Lacroix et al., Comparative Performance of Cyclic Versus Linear Peptides In An ELISA For HIV-1 And HIV-2 Specific Antibodies, No. 3147, June 1989 AIDS Conference, Montreal, Canada; and WO 89/03344. This invention is directed to improvements in such cyclic peptides.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a novel series of peptides which are particularly adapted for detecting 100% of HIV-1 and HIV-2 antibodies and which are capable of detecting such antibodies even when present in very low levels in sera.

More specifically, the novel peptides of the present invention are selected from substantially pure peptides of formulae I or II:



(I)

wherein:

x is independently selected from one of the following amino acid sequence analogs of the amino acid sequence of gp41-HIV-1:

KILAVERYLKDQQLLGIWG- (586-604)

KKILAVERYLKDQQLLGIWG- (585-604),

amino acid sequences corresponding thereto, which sequences are derived from homologous regions of other HIV-1 isolates, and amino acid sequences differing from the above as a result of conservative substitutions, such amino acid sequences being characterized by at least one of a lysine at position 586 or a lysine at both positions 585 and 586;

y, if present, is independently selected from the group consisting of:

-T
-TT
-TTA
-TTAV
-TTAVP
-TTAVPW
-TTAVPWN
-TTAVPWNA
-TTAVPWNAS
-TTAVPWNASW
-TTAVPWNASWS
-TTAVPWNASWSN
-TTAVPWNASWSNK
-TTAVPWNASWSNKS
-TTAVPWNASWSNKSL
-TTAVPWNASWSNKSLE
-TTAVPWNASWSNKSLEQ
-TTAVPWNASWSNKSLEQI,

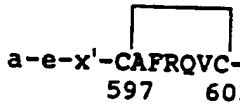
amino acid sequences corresponding thereto, which sequences are derived from homologous regions of other HIV-1 isolates, and amino acid sequences differing from the above as a result of conservative substitutions;

e and f, if present, are independently selected from the group consisting of an amino acid sequence of any one of the epitopes of the region spanning amino acids 586 to 629 of gp41 of HIV-1 or of

the region spanning amino acid sequence 578 to 613 of gp36 of HIV-2, amino acid sequences corresponding thereto and being derived from homologous regions of other HIV-1 or HIV-2 isolates, amino acid sequences differing from the above as a result of conservative substitutions, and any combination of these epitopes;

a is an amino terminus or a substitutent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties; and

b is a carboxy terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties; and



a-e-x'-CAFRQVC-y'-f-b (II)

597 603

wherein:

x' is independently selected from one of the following amino acid sequence analogs of the amino acid sequence of gp36-HIV-2:

KVTAIEKYLQDQARLNSWG (578-596)

KKVTAIEKYLQDQARLNSWG (577-596),

amino acid sequences corresponding thereto, which sequences are derived from homologous regions of other HIV-2 isolates and amino acid sequences differing from the above as a result of conservative substitutions, such sequences being characterized by at least one of a lysine at position 578 or a lysine at both positions 577 and 578;

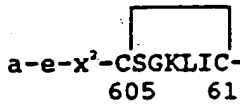
y', if present, is independently selected from the group consisting of:

-H
-HT
-HTT
-HTTV
-HTTVP
-HTTVPW
-HTTVPWV
-HTTVPWVN
-HTTVPWVND
-HTTVPWVNDS,

amino acid sequences corresponding thereto, which sequences are derived from homologous regions of other HIV-2 isolates, and amino acid sequences differing from the above as a result of conservative substitutes; and

e, f, a and b are as defined above.

In another embodiment, the novel peptides of this invention are selected from substantially pure peptides of the formulae III and IV:



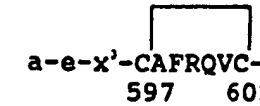
(III)

wherein x', if present, is independently selected from the group consisting of:

G-
WG-
IWG-
GIWG-
LGIWG-
LLGIWG-
QLLGIWG-
QQLLGIWG-
DQQLLGIWG-
KDQQLLGIWG-
LKDQQLLGIWG-
YLKDQQLLGIWG-
RYLKDQQLLGIWG-
ERYLKDQQLLGIWG-
VERYLKDQQLLGIWG-
AVERYLKDQQLLGIWG-
LAVERYLKDQQLLGIWG-
ILAVERYLKDQQLLGIWG-
RILAVERYLKDQQLLGIWG-,

amino acid sequences corresponding thereto, which sequences are derived from homologous regions of other HIV-1 isolates, and amino acid sequences differing from the above as a result of conservative substitutions; and

y,e,f,a and b are as previously defined, one or both of e or f being present; and



(IV)

wherein x', if present, is independently selected from the group consisting of:

G-
WG-
SWG-
NSWG-
LNSWG-
RLNSWG-
ARLNSWG-
QARLNSWG-
DQARLNSWG-
QDQARLNSWG-
LQDQARLNSWG-
YLQDQARLNSWG-
KYLQDQARLNSWG-
EKYLQDQARLNSWG-
IEKYLQDQARLNSWG-
AIEKYLQDQARLNSWG-
TAIEKYLQDQARLNSWG-
VTAIEKYLQDQARLNSWG-
RVTAIEKYLQDQARLNSWG-,

amino acid sequences corresponding thereto, which sequences are derived from homologous regions of other HIV-2 isolates, and amino acid sequences differing from the above as a result of conservative substitutions; and

y', e, f, a and b are as previously defined, one or both of e or f being present.

A particularly preferred peptide of formula I is BCH-408 which has the following sequence:

This peptide incorporates at the f position 619 the amino acid sequence of the epitope located at position

606-610 (SGKLI) of gp41 of HIV-1 and at position 586 a lysine.

A particularly preferred peptide of formula II is BCH-417 which has the following sequence:

This peptide incorporates at the f position 613 the amino acid sequence of the epitope located at position 598-602 (AFRQV) of gp36 of HIV-2 and at position 578 lysine.

DETAILED DESCRIPTION OF THE INVENTION

Selection of peptides for synthesis

The peptides of this invention were synthesized on the basis of published amino acid sequences of HIV-1 and HIV-2. However, it should be understood that sequences derived from the homologous regions of other HIV-1 or HIV-2 isolates can be used without departing from the scope of this invention.

Epitopes in these native sequences were chosen for use as e and f, in the peptides of this invention using various physicochemical principles that aid in predicting which portions of a polypeptide are most likely to be surface oriented and therefore immunogenic. These include the hydrophilicity plots of Hopp and Woods (Proc. Natl. Acad. Sci. 78, 3824-3828, 1981), and a similar approach by Kyte and Doolittle (J. Mol. Biol. 157, 105-132, 1982). Also, the empirical prediction of protein conformation (Chou and Fasman, Ann. Rev. Biochem. 47, 251-276, 1978) is a useful guide in predicting which parts of the polypeptide are likely to be immunogenic.

The e and f epitopes of the peptides of this invention include, but are not limited to, WGCAF (identified by Norrby et al., AIDS Research and Human Retroviruses, Vol. 5, No. 5, 1989); KD, SGKL, and LEDQ (identified by Norrby et al., AIDS, Vol. 3, No. 1 (1989); LKDQ, CSGKLI, and IWG (identified by Mathiesen et al., Immunology, 67 1-7 (1989); and ARILAVERYLKD, and SGKLICTTAVPWNAS (identified by Dopel et al., Jol. of Vir. Meth., 25 167-178 (1989).

It is also within the scope of this invention to modify the peptides of this invention, in order to make them more useful as immunodiagnostic reagents without changing their antigenic properties. Such changes include:

- addition of a cysteine residue at the amino or carboxy terminus in order to facilitate coupling of the peptide to a carrier protein with heterobifunctional cross-linking reagents such as sulfosuccinimidyl-4(p-maleimidophenyl) butyrate, a preferred reagent for effecting such linkages;

- addition of certain amino acids at the amino or carboxy terminus to facilitate linking of peptides to each other, for coupling to a support or larger peptide or for modifying the physical or chemical properties of the peptide. Such changes may be effected, for example, by additions of tyrosine, glutamic acid or aspartic acid, which can be used as linkers via an esterification reaction, and lysine which can be connected by Schiff base or amide formation; and

- derivatization by amino terminal acylation, thioglycolic acid amidation, and carboxy terminal amidation, e.g. using ammonia, methylamine. These modifications result in changes in net charge on the peptide and can also facilitate covalent linking of the peptide to a solid support, a carrier or another

peptide. Th se modifications are not likely t result in immunoreactivity changes to the peptide.

The peptides of this invention may also be modified by various changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use. These changes include preferably the following conservative changes: gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; phe, tyr; ala, ser; ala, thr; ala, val; ala, pro; ala, glu; leu, gln; gly, phe; ile, ser; and ile, met. Methionine, an amino acid which is prone to spontaneous oxidation, can also usually be replaced by norleucine without changing antigenicity.

It may also be convenient to add a "tail" consisting of a small number (1-10) of hydrophobic amino acids to the peptides of this invention. Such tails may facilitate passive adsorption of a peptide to a solid support. This modification can be made at either the COOH or NH, termini. The preferred addition is phe-ala-phe-ala-phe.

In accordance with this invention, the preferred cyclic peptides of formula I are those having x, y, e and f defined as follows:

x: KILAVERYLKDQQLLGIWG, y: TTAVPWNAS, e and f not present (BCH-87ck);

x: KKILAVERYLKDQQLLGIWG, y: TTAVPWNAS, e and f not present (BCH-266); and

x: KILAVERYLKDQQLLGIWG, y: TTAVPWNA,

f: SGKLI and e not present (BCH-408), BCH-408 being the most preferred.

The preferred cyclic peptides of formula II are those having x', y', e and f defined as follows:

x': KVTAIEKYLQDQARLNSWG, y': HTTVPWVNDS and e and f not pres nt (BCH-202ck);

x': KKVTAIEKYLQDQARLNSWG, y': HTTVPWVNDS and e and f not present (BCH-265); and
x': KVTAIEKYLQDQARLNSWG, y': HTTVPWVNDS and
f: AFRQV and e not present (BCH-417), BCH-417 being the most preferred.

TABLE I provides the full amino acid sequences of these preferred peptides (disregarding possible a and b):

TABLE 1 -- Peptide sequences

HIV-1:

BCH-87ck: KILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS

BCH-266: KKILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS

BCH-408: KILAVERYLKDQQLLGIWGCSGKLICTTAVPWNASGKLI

HIV-2:

BCH-202ck: KVTAIEKYLQDQARLNSWGCAFRQVCHTTVPWVNDS

BCH-265: KKVTAIEKYLQDQARLNSWGCAFRQVCHTTVPWVNDS

BCH-417: KVTAIEKYLQDQARLNSWGCAFRQVCHTTVPWVNDSAFRQV

Preparation of linear and cyclic peptides

The peptides of this invention are preferably prepared using c nventional solid phase synthesis.

However, other well known methods of peptide synthesis may also be used. The resin support is any suitable resin conventionally employed in the art for solid phase preparation of polypeptides, preferably p-benzyloxyalcohol polystyrene and p-methylbenzydrylamine resin. Following the coupling of the first protected amino acid to the resin support, the amino protecting group is removed by standard methods conventionally employed in the art of solid phase peptide synthesis. After removal of the amino protecting group, remaining α-amino protected and, if necessary, side chain protected amino acids are coupled, sequentially, in the desired order to obtain the product. Alternatively, multiple amino acid groups may be coupled using solution methodology prior to coupling with the resin-supported amino acid sequence.

The selection of an appropriate coupling reagent follows established art. For instance, suitable coupling reagents are N,N'-diisopropylcarbodiimide or N,N'-dicyclohexylcarbodiimide (DCC) either alone or preferably in the presence of 1-hydroxybenzotriazole. Another useful coupling procedure makes use of preformed symmetrical anhydrides of protected amino acids.

The necessary α-amino protecting group employed for each amino acid introduced onto the growing polypeptide chain is preferably 9-fluorenylmethyloxycarbonyl (Fmoc), although any other suitable protecting group may be employed as long as it does not degrade under the coupling conditions and is readily removable selectively in the presence of any other protecting groups already present in the growing molecule.

The criteria for selecting protecting groups for the side chain amino acids are: (a) stability of

the protecting group to the various reagents und r reaction conditions selective for the removal of the α-amino protecting group at each step of the synthesis: (b) retention of the protecting group's strategic properties (i.e. not be split off under coupling conditions) and (c) removability of the protecting group upon conclusion of the polypeptide synthesis and under conditions that do not otherwise affect the polypeptide structure.

The fully protected resin-supported peptides are cleaved from the p-benzyloxy alcohol resin with 50 to 60 percent solution of trifluoroacetic acid in methylene chloride for 1 to 6 hours at room temperature in the presence of appropriate scavengers such as anisole, thioanisole, ethyl methyl sulfide, 1,2-ethanedithiol and related reagents. Simultaneously, most acid labile side-chain protecting groups may then be removed. More acid resistant protecting groups are removed by HF treatment.

Cyclic peptides of this invention are prepared by the direct oxidative conversion of protected or unprotected SH-groups to a disulfide bond by following techniques generally known in the art of peptide synthesis. The preferred method involves the direct oxidation of free SH-groups with potassium ferricyanide. Such cyclic peptides are believed to assume a more rigid conformation which may favor binding to HIV antibodies.

Peptide mixtures and polymers

Within the scope of this invention are larger peptides formed by the covalently linking of one or more peptides of this invention. Polymers (both homo and co) of these peptides are also envisioned.

Also within the sc pe of the present invention are other cyclic and mixtures of the cyclic peptides of this invention and other cyclic and linear HIV derived peptides. These mixtures have surprisingly been found to provide high sensitivity detection of HIV-1 and HIV-2 antibodies present in serially diluted serum samples and in seroconversion panels (HIV-1). Also it has been found that these mixtures provide a high level of specificity resulting in a minimal number of false positives.

Such mixtures comprise at least one cyclic peptide of the general formulae I or III (preferably BCH-87ck, BCH-266 or BCH-408 and more preferably BCH-408) in combination with at least one cyclic peptide of the general formulae II or IV (preferably BCH-202ck, BCH-265 or BCH-417 and more preferably BCH-417).

HIV antibody detection

The peptides and the peptide mixtures of the present invention are useful as diagnostic reagents for the detection of AIDS-associated antibodies in accordance with methods well-known in the art. These include ELISA, hemagglutination, single-dot and multi-dot methods and assays. The main advantage of the present peptides in the determination of antibodies against AIDS resides in their specificity and high sensitivity, and particularly their ability to detect the presence of very low levels of AIDS infection, when compared with known antigens used so far.

According to one method for the determination of antibodies against HIV-1 or HIV-2, the so-called "Western Blotting" analysis is used [Towbin, H., Staehelin, T. and Gordon, J., Proc. Nat. Acad. Sci. USA 76, 4350-4354 (1979)]. According to this technique

a peptide or peptides of th present invention is (or are) applied to nitrocellulose paper. The nitrocellulose paper is saturated and then treated with the serum to be tested. After washing, the nitrocellulose paper is treated with an anti-human IgG labeled with an enzyme. The enzymatic activity is then determined by a suitable substrate. Of course, other labels, like radioactive or fluorescence labels, may be used.

A preferred convenient and classical technique for the determination of antibodies against HIV-1 or HIV-2 using a peptide or a peptide mixture of the present invention is an enzyme-linked immunosorbent assay (ELISA). In this assay, for example, a peptide, peptide mixture or combination of the present invention is adsorbed onto, or covalently coupled to, the wells of a microtiter plate. The wells are then treated with the sera or analyte to be tested. After washing, anti-human IgG or antihuman IgM labeled with peroxidase is added to the wells. The determination of the peroxidase is performed with a corresponding substrate, e.g., with o-phenylene diamine. Without departing from the usefulness of the illustrative assay, the peroxidase can be exchanged by another label, e.g., by a radioactive, fluorescence chemiluminescence or infra-red emitting label.

In the ELISA test, it is possible to use individual peptides or a combination thereof. The latter is preferable since it allows one to combine the most effective peptides for detecting antibodies while at the same time excluding those that contribute to false responses. It was discovered during the course of these studies that some serum samples gave correct positive results with mixtures of peptides while giving equivocal responses with individual peptides as

antigen. Thus the most preferred test for HIV-1 and HIV-2 antibodies is achieved in accordance with this invention using a combination of peptide antigens.

Another method for the determination of antibodies against HIV-1 or HIV-2 with the peptides or mixture of peptides of this invention is an enzyme immunological test according to the so-called "Double-Antigen-Sandwich-Method". This method is based on the work of Maiolini as described in Immunological Methods 20, 25-34 (1978). According to this method, the serum or other analyte to be tested is contacted with a solid phase on which a peptide or mixture of peptides of the present invention has been coated (capture layer) and with a peptide or a peptide mixture of the present invention which is labeled with peroxidase (probe layer). The immunological reaction can be performed in one or two steps. If the immunological reaction is performed in two steps, then a washing step is preferably performed between the two incubations. After the immunological reaction or reactions, a washing step may also be performed. Thereafter, the peroxidase is determined with a substrate, e.g., with o-phenylene diamine. Other enzymes and chromogens, including those already described can also be employed in this assay.

Suitable solid phases are organic and inorganic polymers, such as amylases, dextrans, natural or modified celluloses, polyethylenes, polystyrenes, polyacrylamides, agaroses, magnetites, porous glass powders, polyvinyldiene fluoride (kynar) and latex, the inner wall of test vessels e.g., test tubes, titer plates or cuvettes of glass or articifial material as well as the surface of solid bodies, e.g., rods of glass and artificial material, rods with terminal thickening, rods with terminal lobes or lamallae.

Spheres of glass and artificial material are especially suitable solid phase carriers.

The peptides and mixtures of peptides of the present invention are not only useful in the determination of antibodies against HIV-1 or HIV-2, but also indirectly for the determination of HIV-1 or HIV-2 itself since these peptides either free, polymerized or conjugated to an appropriate carrier are useful in eliciting antibodies, in particular monoclonal antibodies, against HIV-1 or HIV-2. Such antibodies can be produced by injecting a mammalian or avian animal with a sufficient amount of a peptide or mixture of peptides of the present invention and recovering said antibodies from the serum of said animals. Suitable host animals for eliciting antibodies include mammals such as rabbits, horses, goats, guinea-pigs, rats, mice, cows, sheep, etc..

Various methods which are generally known can be employed using the peptides of this invention or mixtures thereof in the quantitative determination of HIV-1 or HIV-2 infection. In one such procedure known amounts of a serum sample to be assayed, radiolabeled cyclic peptide of the present invention or mixtures of those peptides and unlabeled peptide or mixture of peptides of the present invention are mixed together and allowed to stand. The antibody/antigen complex is separated from the unbound reagents by procedures known in the art, i.e., by treatment with ammonium sulfate, polyethylene glycol, a second antibody either in excess or bound to an insoluble support, dextran-coated charcoal and the like. The concentration of the labeled peptide or mixture of peptides of the present invention is determined in either the bound or unbound phase and the HIV-1 or HIV-2 content of the sample can then be determined by comparing the l vel of label d

component observed t a standard curve in a manner known 'per se'.

Another suitable quantitative method is the "Double-Antibody-Sandwich-Assay". According to this assay the sample to be tested is treated with two different antibodies raised against a peptide of this invention or mixture thereof using different animals, e.g. sheep or rabbits. Alternatively, monoclonal antibodies may be prepared using the well-known Koehler and Milstein technique for producing monoclonal antibodies. In order to distinguish monoclonal antibodies which are directed against the same antigen, but against different epitopes, the method of Stähli et al. [J. of Immunological Methods 32, 297-304 (1980)] can be used. It is also appropriate to use a polyclonal antiserum and a monoclonal antibody.

One of these antibodies is labeled and the other is coated on a solid phase. The suitable solid phases are those mentioned earlier in this application. Suitable labels are enzymes, e.g. peroxidase, radioactive labels or fluorescence-labels. The preferred solid phase is a plastic bead and the preferred label is horse-radish peroxidase.

The sera sample is then incubated with the solid phase antibody and the labeled antibody. It is possible to treat the sample first with the solid phase antibody and after washing to treat the sample with the labeled antibody. However, it is also possible to treat the sample first with the solid phase antibody and after a certain time with the labeled antibody. Preferably the sample is treated together with the solid phase and the labeled antibody.

After the immunological reaction(s), a washing step may be performed. After washing, the label is determined according to procedures known in

the art. In the case where peroxidase is used as the label, the determination is performed with the substrate, e.g., with o-phenylene diamine or with tetramethylbenzidine. The amount of the labeled component is proportional to the amount of the antigen(s) present in the sample.

The methods and assays for the determination and quantification of HIV-1, HIV-2 or of antibodies against HIV-1 or HIV-2 as described above can be conducted in suitable test kits comprising, in a container, a cyclic peptide of the present invention, peptide mixtures or a combination thereof, or antibodies against HIV-1 or HIV-2 elicited by a cyclic peptide or a mixture of cyclic and linear peptides of the present invention.

The peptides of this invention and mixtures and combinations thereof are also useful as the active component of vaccines capable of inducing protective immunity against the HIV-1 and HIV-2 in hosts susceptible to infection with that virus. Routes of administration, antigen doses, number and frequency of injections will vary from individual to individual and may parallel those currently being used in providing immunity to other viral infections. For example, the vaccines are pharmaceutically acceptable compositions containing at least one peptide of this invention, its analogues or mixtures or combinations thereof, in an amount effective in the mammal, including a human, treated with that composition to raise antibodies sufficient to protect the treated mammal from HIV-1 or HIV-2 infection for a period of time.

The vaccines are prepared in accordance with known methods. The vaccine compositions of this invention are conveniently and conventionally combined with physi logically acceptabl carri r materials, such

as pharmaceutical grade saline, tetanus toxoid, and keyhole limpet hemocyanin. The vaccine compositions of this invention may also contain adjuvants or other enhancers of immune response, such as alum preparations, liposomes or immunomodulators. Furthermore, these vaccine compositions may comprise other antigens to provide immunity against other viruses (e.g., HTLV-I and HTLV-II, cytomeglo virus) or pathogens in addition to HIV-1 and HIV-2. The amount of these other antigens is again dependent on the mammal to be treated and the course of the disease. However, the antigen should be present in an amount effective to raise antibodies sufficient to protect the treated mammal from that pathogen or virus for a period of time.

Panel Of Sera Tested

To demonstrate the surprising sensitivity and specificity of the peptides of this invention a panel of sera was tested with illustrative peptides.

O.D. Values were obtained at 450 nm and the blank values measured with the sample dilution buffer were not subtracted.

Samples NEIA-2*2, BBI-1-162 to 168, 87B140, 87L139, 87V103 are all negative for HIV antibodies. Sample LSPQ-S9-1 is an early seroconverter (HIV-1). The series labeled CAP-113 to CAP-120 corresponds to a pool of seven HIV-1 positive plasma samples serially diluted with an HIV-negative plasma. CAP-113 is the pool diluted 50-fold with the HIV-negative plasma; CAP-114 is diluted 100-fold; CAP-115 is diluted 200-fold; etc. Similarly, the series labeled CAP-222 to CAP-230 corresponds to a pool of seven HIV-2 confirmed seropositive plasma samples. CAP-222 is diluted by 50 with an HIV-negative plasma; CAP-223 by 100; etc. Before the assay is don , ach sample, including the

CAP-series, is further diluted by 50 with the sample dilution buffer. In these tests, the cut-off for seropositivity is defined as the sum of the O.D. value for sample NEIA-2*2 plus 0.100.

Results

The cyclic peptides of the present invention were coated and tested in accordance with the ELISA test described previously. TABLE 2 compares the sensitivity of peptide BCH-87c (586(arginine)) to the sensitivity of peptide BCH-87ck (586(lysine)) and peptide BCH-265 (586(lysine) - 585(lysine)) at progressively higher dilutions of the antibody in the sera samples. It was found that the substitution of a lysine for the arginine at amino acid position 586 increased the sensitivity in detecting HIV-1 antibodies. A further increase in sensitivity was obtained by an additional lysine at position 585. TABLE 3 compares the activity of BCH-87c, BCH-87ck, BCH-266 and BCH-408 at progressively higher dilutions. It is evident from TABLE 3 that peptide BCH-408, wherein an important epitope located at position 606-610 (SGKLI) is repeated at its c-terminus, has superior sensitivity compared to the other peptides.

It was found with the HIV-2 peptides that substituting a lysine for the arginine at amino acid position 578 increased the sensitivity in detecting HIV-2 antibodies. A further increase in sensitivity was obtained by an additional lysine at position 577. TABLE 4 compares the sensitivity of the peptide BCH-202c (578(arginine)) to the sensitivity of peptide BCH-202ck (578(lysine)) and peptide BCH-266 (598(lysine) - 577(lysine)) at progressively higher dilutions of the antibody in the sera samples.

Peptide cocktails were also made to detect a mixture of HIV-1 and HIV-2 antibodies. TABLE 5 illustrates the sensitivity of peptide cocktail mixtures BCH-87c and BCH-202c (arginine) versus BCH-87ck and BCH-202ck (lysine). The peptide cocktail which included the peptides with lysine substituted for arginine have a higher sensitivity in detecting HIV antibodies.

TABLE 2 - HIV-1

Test 314		O.D. 450 nm		
	Sample ID	BC-87c	BCH-87ck	BCH-266
	Dil. Buffer	0.017	0.016	0.014
5	NEIA-2*2	0.045	0.050	0.069
	BBI-1-162	0.010	0.015	0.028
	BBI-1-169	0.095	0.090	0.138
	BBI-1-172	0.012	0.019	0.030
	CAP-113	>2.8	>2.8	>2.8
10	CAP-114	>2.8	>2.8	>2.8
	CAP-115	1.935	2.593	>2.8
	CAP-116	1.275	1.871	2.376
	CAP-117	0.739	1.158	1.569
	CAP-118	0.433	0.679	0.934
15	CAP-119	0.231	0.392	0.567
	CAP-120	0.137	0.229	0.376
	CAP-222	0.059	0.069	0.095
	CAP-223	0.053	0.060	0.075
	CAP-224	0.051	0.054	0.084
20	CAP-225	0.049	0.050	0.135
	CAP-226	0.046	0.050	0.140
	CAP-227	0.041	0.043	0.085
	CAP-228	0.038	0.042	0.091
	CAP-230	0.043	0.049	0.123

TABLE 3 - HIV-1

Test 350

O.D. 450 nm

Sample ID	BCH-87c	BCH-87ck	BCH-266	BCH-408
Dil.Buffer	0.016	0.032	0.013	0.015
NEIA-2*2	0.027	0.034	0.023	0.035
2-87-V-103	0.020	0.015	0.020	0.014
2-87-L-139	0.158	0.409	0.147	0.025
89-D-307	0.026	0.022	0.030	0.029
CAP-10	>2.8	>2.8	>2.8	>2.8
CAP-11	>2.8	>2.8	>2.8	>2.8
CAP-12	2.432	2.735	>2.8	>2.8
CAP-13	1.488	1.676	2.021	2.319
CAP-14	0.866	1.089	1.248	1.433
CAP-15	0.449	0.643	0.680	0.8[illegible]
CAP-16	0.262	0.274	0.366	0.455
CAP-17	0.141	0.191	0.198	0.244
CAP-18	0.099	0.114	0.116	0.156
CAP-19	0.068	0.053	0.056	0.1[illegible]

TABLE 4 - HIV-2

Test 226	O.D. 450 nm		
Sample ID	BC-202c	BCH-202ck	BCH-265
Dil. Buffer	0.015	0.014	0.015
NEIA-2*2	0.020	0.027	0.022
BBI-1-162	0.018	0.033	0.059
BBI-1-169	0.018	0.028	0.076
BBI-1-172	0.019	0.024	0.050
CAP-113	1.795	>2.8	>2.8
CAP-114	1.070	2.015	2.134
CAP-115	0.611	1.195	1.330
CAP-116	0.325	0.642	0.718
CAP-117	0.174	0.365	0.410
CAP-118	0.089	0.189	0.190
CAP-119	0.057	0.082	0.117
CAP-120	0.041	0.084	0.094
CAP-222	>2.8	>2.8	>2.8
CAP-223	>2.8	>2.8	>2.8
CAP-224	2.313	>2.8	>2.8
CAP-225	1.448	2.418	2.480
CAP-226	0.758	1.436	1.674
CAP-227	0.448	0.745	0.960
CAP-228	0.233	0.409	0.363
CAP-230	0.139	0.279	0.272

TABLE 5 - HIV-1

Comparative Performance of Two Peptide-Cocktails

Test 310	O.D. 450 nm	
Sample ID	BC-87c BC-202c	BCH-87ck BCH-202ck
Dil. Buffer	0.015	0.013
NEIA-2*2	0.034	0.062
BBI-1-162	0.044	0.034
BBI-1-163	0.032	0.042
BBI-1-164	0.052	0.077
BBI-1-165	0.038	0.059
BBI-1-166	0.045	0.081
BBI-1-167	0.020	0.064
BBI-1-168	0.031	0.041
87-B-140	0.005	0.015
87-L-139	0.025	0.068
87-V-103	0.022	0.035
CAP-113	>2.8	>2.8
CAP-114	2.634	>2.8
CAP-115	1.720	>2.8
CAP-116	0.965	2.459
CAP-117	0.548	1.685
CAP-118	0.302	1.036
CAP-119	0.158	0.609
CAP-120	0.104	0.34
CAP-222	>2.8	>2.8
CAP-223	2.470	>2.8
CAP-224	1.709	>2.8
CAP-225	1.003	2.441
CAP-226	0.554	1.733
CAP-227	0.289	1.001
CAP-228	0.169	0.611
CAP-230	0.086	0.343
LSPQ-89-1	0.123	1.273
BBI-A-01	0.067	0.072
BBI-A-02	0.114	0.130
BBI-A-03	0.180	1.069
BBI-A-04	2.401	>2.8
BBI-A-05	>2.8	>2.8
BBI-A-06	>2.8	>2.8
BBI-A-07	>2.8	>2.8
BBI-A-08	>2.8	>2.8
BBI-A-09	>2.8	>2.8
BBI-C-20	0.019	0.050
BBI-C-21	0.017	0.048
BBI-C-22	0.030	0.121
BBI-C-24	0.210	1.748
BBI-C-25	0.372	2.452
BBI-C-26	0.374	2.353

Th following illustrates the general procedures for the synthesis and utilization of the peptides of this invention.

Procedure 1: Preparation of Resins Carrying the Nα-FMOC Protected Amino Acid Residue

The desired Nα-FMOC protected amino acid residue in a mixture of methylene chloride (CH_2Cl_2) and dimethylformamide (DMF) (4:1) was added to a suspension of p-benzyloxy alcohol resin in CH_2Cl_2:DMF (4:1) at 0°C. The mixture was stirred manually for a few seconds and then treated with N,N'-dicyclohexylcarbodiimide (DCC) followed by a catalytic amount of 4-(dimethylamino) pyridine. The mixture was stirred at 0°C for an additional 30 minutes and then at room temperature overnight. The filtered resin was washed successively with CH_2Cl_2, DMF and isopropanol (3 washes each) and finally, with CH_2Cl_2. The resin was suspended in CH_2Cl_2, chilled in an ice bath and redistilled pyridine was added to the stirred suspension Benzoyl chloride was then also added. Stirring was continued at 0°C for 30 minutes and then at room temperature for 60 minutes. After filtration, the resin was washed successively with CH_2Cl_2, DMF and isopropanol (3 washes each) and finally with petroleum ether (twice) before being dried under high vacuum to a constant weight. Spectrophotometric determination of substitution according to Meienhofer et al. (Int. J. Peptide Protein Res., 13, 35, 1979) indicates the degree of substitution on the resin.

Procedure 2: Coupling of Subsequent Amino Acids

The resin carrying the Nα-FMOC protected first amino acid residue was placed in a reaction vessel of a Biosearch 9600 Peptide Synthesizer and tr ated as follows:

1) Washed with DMF (4 times for 20 sec. each)
2) Prewashed with a 30% solution of piperidine in DMF (3 min.)
3) Deprotected with a 30% solution of piperidine in DMF (7 min.)
4) Washed with DMF (8 times for 20 sec. each)
5) Checked for free amino groups - Kaiser Test (must be positive)
6) The peptide resin was then gently shaken for 1 or 2 hrs with 8 equivalents of the desired FMOC-protected amino acid and 1-hydroxybenzotriazole and benzotriazol-1-yl-oxy-tris(dimethyl-amino)phosphonium hexafluorophosphate all dissolved in dry redistilled DMF containing 16 equivalents of 4-methylmorpholine.
7) Washed with DMF (6 times for 20 sec. each)

After step 7, an aliquot was taken for a ninhydrin test. If the test was negative, one goes back to step 1 for coupling of the next amino acid. If the test was positive or slightly positive, steps 6 and 7 should be repeated.

The above scheme maybe used for coupling each of the amino acids of the peptides described in this invention. Nα-protection with FMOC may also be used with each of the remaining amino acids throughout the synthesis.

Radiolabeled peptides may be prepared by incorporation of a tritiated amino acid using the above coupling protocol.

After the addition of the last amino acid, the Nα-FMOC of the N-terminal residue is removed by going back to steps 1-7 of the above scheme. The peptide resin is washed with CH_2Cl_2 and dried <u>in vacuo</u> to give the crude protected peptide.

Procedure 3: Deprotection and Cleavage of the Peptides from the Resin

The protected peptide-resin was suspended in a 55% solution of trifluoroacetic acid (TFA) in CH_2Cl_2, containing 2.5% ethanedithiol and 2.5% anisole. The mixture was flushed with N_2 and stirred for 1.5 hours at room temperature. The mixture was filtered and the resin washed with CH_2Cl_2. The resin was treated again with 20% TFA in CH_2Cl_2 for 5 minutes at room temperature. The mixture was filtered and the resin washed with 20% TFA in CH_2Cl_2 and then washed with CH_2Cl_2. The combined filtrates were evaporated in vacuo below 35°C and the residue triturated several times with dry dimethyl ether. The solid was dissolved in 10% aqueous acetic acid and lyophilized to afford the crude product.

The peptides containing arg and cys residues are further deprotected by HF treatment at 0°C for 1 hour in the presence of anisole and dimethylsulfide. The peptides were extracted with 10% aqueous acetic acid, washed with dimethyl ether and lyophilized to afford the crude peptides.

Procedure 4: Purification of Peptides

The crude peptides were purified by preparative HPLC on a Vydac column (2.5 X 25 mm) of C_{18} or C_4 reverse phase with a gradient of the mobile phase. The effluent was monitored at 220 nm and subsequently by analytical HPLC. Relevant fractions were pooled, evaporated and lyophilized. The identity of the synthetic peptides was verified by analytical reverse phase chromatography and by amino acid analysis.

Procedure 5: Cyclization of Peptids

A solution of potassium ferricyanide (0.01M, pH 7.0) was added slowly to a dilute aqueous solution (0.5 mM) of the linear peptide at pH 7.0. After 24 hours at room temperature, the pH was lowered to 5.0 and the solution treated with ion exchange resin (Bio-Rad Ag-3-X4a, Cl-form) for 30 minutes. The suspension was filtered and the filtrate lyophilized to give the crude cyclic peptide. The peptide was purified by preparative reverse phase HPLC and characterized by amino acid analysis. Proof of cyclicity was obtained by comparing the HPLC mobility of the cyclic peptide with the starting linear peptide by reducing an aliquot of the cyclic peptide back to the linear peptide and also by observing the disappearance of free sulfhydryl groups (Ellman's Test) after the cyclization.

Procedure 6: Conjugation of Peptides to Bovine Serum Albumin or Keyhole Limpet Hemocyanin

Peptides were conjugated to BSA or KLH previously derivatized with either sulfosuccinimidyl 4-(p-maleimidophenyl) butyrate (Sulfo-SMPB) or sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC).

An aqueous solution of sulfo-SMPB or sulfo-SMCC (Pierce Chemicals) was added to a solution of BSA or KLH in 0.02 M sodium phosphate buffer (pH 7.0). The mixture was shaken at room temperature for 45 minutes and the activated carrier immediately applied to a Sephadex G-25 column equilibrated with 0.1M sodium phosphate buffer (pH 6.0) at 4°C.

The fractions of the first peak absorbance (280 nm) corresponding to activated carrier were combined in a round bottom flask t which was added a solution of peptide in 0.05 M sodium phosphat buffer

(pH 6.2). The mixture was thoroughly flushed with N_2 and incubated overnight at room temperature. The coupling efficiency was monitored using ^3H-labeled peptide and by amino acid analysis of the conjugate.

Procedure 7: Detection of Antibodies to HIV by an Enzyme Linked Immunosorbent Assay (ELISA)

Each well of the microtiter plate is saturated with 100µl of a solution containing a peptide or mixture of peptides (5 µg/ml) and left overnight. The wells are emptied and washed twice with a washing buffer (Tris, 0.043M; NaCl, 0.5M; thimerosal, 0.01% w/v; Tween 20, 0.05% v/v; pH 7.4). The wells are then saturated with 0.35 ml of washing buffer for 1 hr. at 37°C and washed once with the same buffer. Serum samples to be analyzed are diluted with specimen buffer (washing buffer plus casein, 0.05% w/v). The wells are rinsed with washing buffer prior to the addition of the diluted serum sample (0.1 ml). These are left to incubate for 1 hr. at room temperature. The wells are then emptied, washed twice rapidly and then once for two minutes with washing buffer. The conjugate solution (affinity purified goat antibody to human IgG peroxidase labeled, 0.5 mg in 5 ml 50% glycerol) diluted with 1% w/v bovine serum albumin in washing buffer is added to each well (0.1 ml) and incubated for 1 hr. at room temperature. The wells are then emptied and washed twice rapidly with washing buffer and then five times in which the buffer was in contact with the well 2 minutes per washing. The substrate solution (3,3',5,5'-tetramethylbenzidine, 8 mg per ml of DMSO) is diluted with 100 volumes 0.1M citrate-acetate buffer, pH 5.6 containing 0.1% v/v of 30% H_2O_2 and added to each well (0.1 ml per well). After 10 minutes the contents of each well is treated with 0.1 ml 2N H_2SO_4

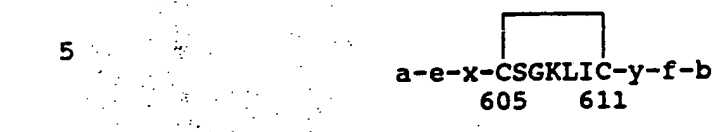
and the optical density read at 450 nm. All determinations are done in duplicate.

Procedure 8: Preparation of Peptide Cocktails

Peptide cocktails were prepared by mixing together equal volumes of two peptide solutions each at 10 ug/ml. One cocktail used peptides BCH-87c and BCH-202c and the other cocktail was a mixture of BCH-87ck and BCH-202ck. Each cocktail was used to coat two series of plates as described earlier.

WE CLAIM:

1. A substantially pure peptide of the formula



where x is independently selected from the group consisting of:

KILAVERYLKDQQLLGIWG (586-604)

KKILAVERYLKDQQLLGIWG (585-604),

amino acid sequences corresponding thereto, which amino acid sequences are derived from homologous regions of other HIV-1 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions, such peptides being characterized by at least one of a lysine at postion 586 or a lysine at both position 585 and 586;

y, if present, is independently selected from the group consisting of:

-T
-TT
-TTA
-TTAV
-TTAVP
-TTAVPW
-TTAVPWN
-TTAVPWNA
-TTAVPWNAS
-TTAVPWNASW
-TTAVPWNASWS
-TTAVPWNASWSN
-TTAVPWNASWSNK
-TTAVPWNASWSNKS
-TTAVPWNASWSNKSL

-TTAVPWNASWSNKSLE
-TTAVPWNASWSNKSLEQ
-TTAVPWNASWSNKSLEQI,

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-1 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions;

e and f, if present, are independently selected from the group consisting of an amino acid sequence of any one of the epitopes of the region spanning amino acids 586 to 629 of gp41 of HIV-1 or of the region spanning amino acid sequence 578 to 613 of gp36 of HIV-2, amino acid sequences corresponding thereto and being derived from homologous regions of other HIV-1 or HIV-2 isolates, amino acid sequences differing from the above as a result of conservative substitutions, and any combination of these epitopes;

a is an amino terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties; and

b is a carboxy terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties.

2. A substantially pure peptide of the formula:

wherein x' is independently selected from the group consisting of:

KVTAIEKYLQDQARLNSWG (578-596)

KKVTAIEKYLQDQARLNSWG (577-596),

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-2 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions, such amino acid sequences being characterized by at least one of a lysine at position 578 or a lysine at both positions 577 and 578;

y', if present, is independently selected from the group consisting of:

-H
-HT
-HTT
-HTTV
-HTTVP
-HTTVPW
-HTTVPWV
-HTTVPWVN
-HTTVPWVND
-HTTVPWVNDS,

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-2 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions;

e and f, if present, are independently selected from the group consisting of an amino acid sequence of any one of the epitopes of the region spanning amino acid sequence 586 to 629 of gp41 of HIV-1 or of the region spanning amino acids 578 to 613 of gp36 of the HIV-2, amino acid sequences corresponding thereto and being derived from homologous regions of other HIV-1 or HIV-2 isolates, amino acid sequences differing from the above as a result of conservative substitutions, and any combination of these epitopes;

a is an amino terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties; and

b is a carboxy terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties.

3. The peptide according to Claim 1, wherein x is KILAVERYLKDQQLLGIWG and y is TTAVPWNAS and e and f are not present.

4. The peptide according to Claim 1, wherein x is KKILAVERYLKDQQLLGIWG and y is TTAVPWNAS and e and f are not present.

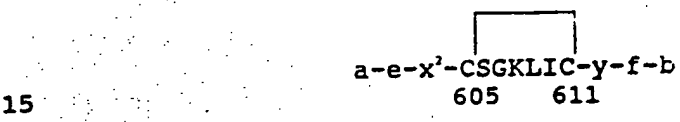
5. The peptide according to Claim 1, wherein x is KILAVERYLKDQQLLGIWG, y is TTAVPWNAS, f is SGKLI and e is not present.

6. The peptide according to Claim 2, wherein x' is KVTAIEKYLQDQARLNSWG and y' is HTTVPWVNDS and e and f are not present.

7. The peptide according to Claim 2, wherein x' is KKVTAIEKYLQDQARLNSWG and y' is HTTVPWVNDS and e and f are not present.

8. The peptide according to Claim 2, wherein x' is KVTAIEKYLQDQARLNSWG and y' is HTTVPWVNDS, f is AFRQV and e is not present.

9. A substantially pure peptide of the formula



where x^2, if present, is independently selected from the group consisting of:

G-
WG-
IWG-
GIWG-
LGIWG-
LLGIWG-
QLLGIWG-
QQLLGIWG-
DQQLLGIWG-
KDQQLLGIWG-
LKDQQLLGIWG-
YLKDQQLLGIWG-
RYLKDQQLLGIWG-
ERYLKDQQLLGIWG-
VERYLKDQQLLGIWG-

AVERYLKDQQLLGIWG-
LAVERYLKDQQLLGIWG-
ILAVERYLKDQQLLGIWG-
RILAVERYLKDQQLLGIWG-,

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-1 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions;

y, if present, is independently selected from the group consisting of:

-T
-TT
-TTA
-TTAV
-TTAVP
-TTAVPW
-TTAVPWN
-TTAVPWNA
-TTAVPWNAS
-TTAVPWNASW
-TTAVPWNASWS
-TTAVPWNASWSN
-TTAVPWNASWSNK
-TTAVPWNASWSNKS
-TTAVPWNASWSNKSL
-TTAVPWNASWSNKSLE
-TTAVPWNASWSNKSLEQ
-TTAVPWNASWSNKSLEQI,

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-1 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions;

e and f are independently selected from the group consisting of an amino acid sequence of any one of the epitopes of the region spanning amino acids 586 to 629 of gp41 of HIV-1 or of the region spanning amino acid sequence 578-613 of gp36 of HIV-2, amino acid sequences corresponding thereto and being derived from homologous regions of other HIV-1 or HIV-2 isolates, amino acid sequences differing from the above as a result of conservative substitutions, and any combination of these epitopes, one or both of e and f being present in the peptide;

a is an amino terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties; and

b is a carboxy terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties.

10. A substantially pure peptide of the formula:

wherein x', if present, is independently selected from the group consisting of:

G-

WG-

SWG-

NSWG-

LNSWG-

RLNSWG-
ARLNSWG-
QARLNSWG-
DQARLNSWG-
QDQARLNSWG-
LQDQARLNSWG-
YLQDQARLNSWG-
KYLQDQARLNSWG-
EKYLQDQARLNSWG-
IEKYLQDQARLNSWG-
AIEKYLQDQARLNSWG-
TAIEKYLQDQARLNSWG-
VTAIEKYLQDQARLNSWG-
RVTAIEKYLQDQARLNSWG-,

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-2 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions;

y', if present, is independently selected from the group consisting of:

-H
-HT
-HTT
-HTTV
-HTTVP
-HTTVPW
-HTTVPWV
-HTTVPWVN
-HTTVPWVND
-HTTVPWVNDS,

amino acid sequences corresponding thereto which amino acid sequences are derived from homologous regions of other HIV-2 is lates and amino acid sequences differing

from the above as a result of conservative amino acid substitutions;

e and f are independently selected from the group consisting of an amino acid sequence of any one of the epitopes of the region spanning amino acid sequence 586 to 629 of gp41 of HIV-1 or of the region spanning amino acids 578 to 613 of gp36 of the HIV-2, amino acid sequences corresponding thereto and being derived from homologous regions of other HIV-1 or HIV-2 isolates, amino acid sequences differing from the above as a result of conservative substitutions, and any combination of these epitopes, one or both of e' and f' being present in said peptide;

a is an amino terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties; and

b is a carboxy terminus or a substituent effective as a coupling agent and/or to make the peptide more useful as an immunodiagnostic reagent without changing its antigenic properties.

11. A mixture comprising two or more peptides selected from the group consisting of the peptides of any one of claims 1, 2, 9 and 10.

12. The mixture according to claim 11 comprising at least the following peptide:

a-KILAVERYLKDQQLLGIWGCSGKLICTTAVPWNASGKLI-b (BCH-408)
586 605 611 619

amino acid sequences corresponding thereto, which amino acid sequences are derived from homologous regions of other HIV-1 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutuions, such amino acid sequences being characterized by a lysine at position 586.

13. The mixture according to claim 11 comprising at least the following peptide:

a-KVTAIEKYLQDQARLNSWCAFRQVCHTTVPWVNDSAFRQV-b (BCH-417)
578 597 603 613

amino acid sequences corresponding thereto, which amino acid sequences are derived from homologous regions of other HIV-2 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutuions, such amino acid sequences being characterized by a lysine at position 578.

14. The mixture according to claim 11 comprising at least the following peptide:

a-KILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS-b (BCH-87ck),
586 605 611 620

amino acid sequences corresponding thereto, which amino acid sequences are derived from homologous regions of other HIV-1 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions, such amino acid sequences being characterized by a lysine at position 586.

15. The mixture according to claim 11 comprising at least the following peptide:

a-KVTAIEKYLQDQARLNSWGCAFRQVCHTTVPWVNDS-b (BCH-202ck),
578 597 603 613

amino acid sequences corresponding thereto, which amino acid sequences are derived from homologous regions of other HIV-2 isolates and amino acid sequences differing from the above as a result of conservative amino acid substitutions, such amino acid sequences being characterized by a lysine at position 578.

16. A means for detecting the presence of antibodies selected from the group consisting of HIV-1 antibodies, HIV-2 antibodies, and mixtures thereof in an analyte, the means being characterized by a peptide according to any one of claims 1 to 10 or by a mixture according to any one of claims 11 to 15.

17. A method for detecting the presence of antibodies selected from the group consisting of HIV-1 antibodies, HIV-2 antibodies and mixtures thereof in an analyte comprising the step of contacting an aliquot of the analyte with a peptide according to any one of claims 1 to 10 or a mixture according to any one of claims 11 to 15.

18. The method according to claim 17 selected from the group consisting of ELISA, hemagglutination, single-dot or multi-dot strip assay methods.

19. An antibody immunologically cross reactive with a peptide according to any one of claims 1-10 and mixtures thereof.

20. The antibody according to claim 19, the antibody being a monoclonal antibody or a mixture of said monoclonal antibodies.

21. A means for detecting the presence of antigens selected from the group consisting of HIV-1 antigens, HIV-2 antigens and mixtures thereof in an analyte, the means being characterized by an antibody according to claim 19 or 20.

22. A method for detecting the presence of antigens selected from the group consisting of HIV-1 antigens, HIV-2 antigens and mixtures thereof in an analyte comprising the step of contacting an aliquot of the analyte with an antibody according to claim 19 or 20.

23. A pharmaceutically acceptable composition comprising a peptide according to any one of claims 1 to 10 or a mixture according to any one of claims 11 to 15, said peptide or said mixture being present in the composition in an amount effective in a mammal, including a human, treated with that composition to raise antibodies sufficient to protect the treated mammal from HIV-1 or HIV-2 viral infections for a period of time.

24. The pharmaceutical composition according to claim 23 including a physiologically acceptable carrier.

25. The pharmaceutical composition according to claim 23 including an adjuvant or enhancer of the immune r sponse.

26. The pharmaceutical composition according to claim 23 including a second antigen to a pathogen other than HIV-1 or HIV-2 virus, said second antigen being present in an amount effective to raise antibodies sufficient to protect the treated mammal from infection by that pathogen for a period of time.

27. A method of protecting a mammal, including a human, from infection by HIV-1, HIV-2 or coinfection with HIV-1 or HIV-2 and another pathogen comprising the step of treating the mammal with a pharmaceutical composition according to any one of claims 23 to 26.

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 91/00233

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 13/00 A 61 K 39/21 G 01 N 33/569

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0326490 (IAF BIOCHEM INTERNATIONAL INC.) 2 August 1989, see the whole document, especially the formulae on pages 4-5,6; lines 57-61; page 7, lines 3-7 ---	1-26
X	WO,A,8903844 (FERRING AB) 5 May 1989, see the whole document, especially claims 1,2, pages 20-21 ---	1-26
A	EP,A,0247557 (HOFFMAN-LA ROCHE & CO. AG) 2 December 1987, see the whole document, especially claims 1,15; pages 8-9 ---	1-26
A	EP,A,0199438 (CHANG et al.) 29 October 1986, see the whole document, especially claim 2, pages 13-14 --- -/-	1-26

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01-10-1991	23 OCT. 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8706005 (ROSEN et al.) 8 October 1987, see the whole document, especially the claims cited in the application	1-26
A	Archives of AIDS Research, vol. II, (Quebec, CA), M. Lacroix et al.:"A Peptide-Based Enzyme Immunoassay for the Detection of Antibodies to HIV", page 65, see the whole disclosure	16-18
A	Chemical Abstracts, vol. 112, no. 7, 12 February 1990, (Columbus, Ohio, US), T.F. Schulz et al.:"Recombinant Peptides Derived from the Env-Gene of HIV-2 in the Serodiagnosis of HIV-2 Infections", see page 544, abstract no. 53318x & AIDS (London), 1989, 3(3), 165-72, see the whole abstract	16-22
X	V Conf. Int. SIDA; June 1989, (Montreal, CA), M. Lacroix et al.:"Comparative Performance of Cyclic Versus Linear Peptides in an Elisa for HIV-1- and HIV-2- Specific Antibodies", see the whole abstract	16-18
X	V Int. Conf. on AIDS, June 1988, (Montreal, CA), M. Lacroix et al.:"A Peptide-Based Microtiter Elisa Kit for the Detection of Antibodies to HIV-1 and HIV-2", see the whole abstract	16-18
X	V Conf. Int. sur le SIDA, June 1989, (Montreal, CA), M. Lacroix et al.:"Sensitive Detection of HIV-1 Antibody with Peptide-Based Elisa in two Seroconversion Panels", see the whole abstract	16-18

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 27 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1 (iv): methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods

2. [ ] Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9100233 SA 48914

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 16/10/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0326490	02-08-89	AU-A-	2851389	27-07-89
		JP-A-	1224397	07-09-89
WO-A- 8903844	05-05-89	AU-A-	2624288	23-05-89
		EP-A-	0396559	14-11-90
		JP-T-	3500648	14-02-91
		SE-A-	8704185	29-04-89
EP-A- 0247557	02-12-87	JP-A-	62294697	22-12-87
		US-A-	4957737	18-09-90
EP-A- 0199438	29-10-86	US-A-	4774175	27-09-88
WO-A- 8706005	08-10-87	AU-A-	7234387	20-10-87
		EP-A-	0261224	30-03-88
		JP-T-	63502904	27-10-88

EPO FORM P0479